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Hantaan/Andes virus DNA vaccine elicits a broadly cross-reactive neutralizing antibody response in nonhuman primates

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Abstract

At least four hantavirus species cause disease with prominent renal involvement-hemorrhagic fever with renal syndrome (HFRS); and several hantavirus strains cause disease with significant pulmonary involvement-hantavirus pulmonary syndrome (HPS). The most prevalent and lethal hantaviruses associated with HFRS and HPS are Hantaan virus (HTNV) and Andes virus (ANDV), respectively. Here, we constructed a DNA vaccine plasmid (pWRG/HA-M) that contains both the HTNV and ANDV M gene segments. Rhesus macaques vaccinated with pWRG/HA-M produced antibodies that bound the M gene products (i.e., G1 and G2 glycoproteins), and neutralized both HTNV and ANDV. Neutralizing antibody titers elicited by the dual-immunogen pWRG/HA-M, or single-immunogen plasmids expressing only the HTNV or ANDV glycoproteins, increased rapidly to high levels after a booster vaccination administered 1–2 years after the initial vaccination series. Memory responses elicited by this long-range boost exhibited an increased breadth of cross-neutralizing activity relative to the primary response. This is the first time that hantavirus M gene-based DNA vaccines have been shown to elicit a potent memory response, and to elicit antibody responses that neutralize viruses that cause both HFRS and HPS.
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Keywords: Hantavirus; DNA vaccine; Hantaan virus; Andes virus; Neutralizing antibodies

Introduction

Hantaviruses are rodent-borne viruses that cause hemorrhagic fever in humans. Different hantaviruses are associated with different disease syndromes with varying degrees of severity. There are at least four hantaviruses associated with hemorrhagic fever with renal syndrome: Hantaan virus (HTNV), Seoul virus (SEOV), Dobrova virus (DOBV), and Puumala virus (PUUV). The most prevalent and lethal HFRS-associated hantavirus is HTNV (>100,000 cases per year, mostly in Asia) with a case-fatality rate of 10–15%. Several hantaviruses are associated with hantavirus pulmonary syndrome (HPS) including Andes virus (ANDV), Sin Nombre virus (SNV), and Black Creek Canal virus (BCCV). There have been over 1900 cases of HPS in the Americas between 1993 and 2004 and ANDV (found in Argentina and Chile) is believed to be the etiologic agent for approximately half of these cases (PAHO, 2004; Tischler et al., 2005). There are no vaccines or specific antiviral drugs licensed by the Food and Drug Administration to treat or prevent HFRS or HPS.

Hantaviruses, family Bunyaviridae, have a genome consisting of three segments of negative-sense RNA. The large (L), small (S), and medium (M) segments encode the RNA-dependent RNA polymerase, nucleocapsid, and surface glycoproteins (G1 and G2), respectively (Schmaljohn and Hooper, 2001). We previously demonstrated that M genome segment-based DNA vaccines expressing either the SEOV, HTNV, or ANDV G1 and G2 glycoproteins elicit high-titer neutralizing antibodies in nonhuman primates (Custer et al., 2003; Hooper et al., 1999, 2001a). Here, we report the construction and testing of a DNA vaccine plasmid (pWRG/HA-M) that contains both the HTNV and ANDV M genome segments. This DNA vaccine was capable of eliciting antibodies that cross-neutralized both HFRS and HPS hantaviruses after the initial vaccination series. Interestingly, a booster vaccination administered >1 year after the initial series increased the cross-neutralizing antibody response. This long-range boost effect on cross-neutralizing antibody was also observed in nonhuman

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14. ABSTRACT

At least four hantavirus species cause disease with prominent renal involvement hemorrhagic fever with renal syndrome (HFRS); and several hantavirus strains cause disease with significant pulmonary involvement- hantavirus pulmonary syndrome (HPS). The most prevalent and lethal hantaviruses associated with HFRS and HPS are Hantaan virus (HTNV) and Andes virus (ANDV), respectively. Here, we constructed a DNA vaccine plasmid (pWRG/HA-M) that contains both the HTNV and ANDV M gene segments. Rhesus macaques vaccinated with pWRG/HA-M produced antibodies that bound the M gene products (i.e., G1 and G2 glycoproteins), and neutralized both HTNV and ANDV. Neutralizing antibody titers elicited by the dual-immunogen pWRG/HA-M,or single-immunogen plasmids expressing only the HTNV or ANDV glycoproteins increased rapidly to high levels after a booster vaccination administered 1-2 years after the initial vaccination series. Memory responses elicited by this long-range boost exhibited an increased breadth of cross-neutralizing activity relative to the primary response. This is the first time that hantavirus M gene-based DNA vaccines have been shown to elicit a potent memory response, and to elicit antibody responses that neutralize viruses that cause both HFRS and HPS.

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Hantaan, hantavirus, Andes virus, pulmonary syndrome, DNA vaccine, efficacy, neutralizing antibody, laboratory animals, nonhuman primates

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primates (NHP) vaccinated with the HTNV or ANDV M segment-based DNA vaccines.

Results

Construction of a DNA vaccine plasmid expressing both the HTNV and ANDV G1 and G2 glycoproteins

A plasmid containing the M genome segments of both HTNV and ANDV was constructed by using pWRG/AND-M (Custer et al., 2003) as template and PCR-amplifying the ANDV M genome segment, flanked by the CMV promoter/intron A at the 5′ end and polyadenylation signal at the 3′ end. This cassette was inserted into the *Xba*I site downstream of the HTNV M genome segment contained in pWRG/HTN-M(x) (Fig. 1A). The resulting plasmid, pWRG/HA-M, was tested for expression of the glycoproteins of HTNV and ANDV by RIPA.

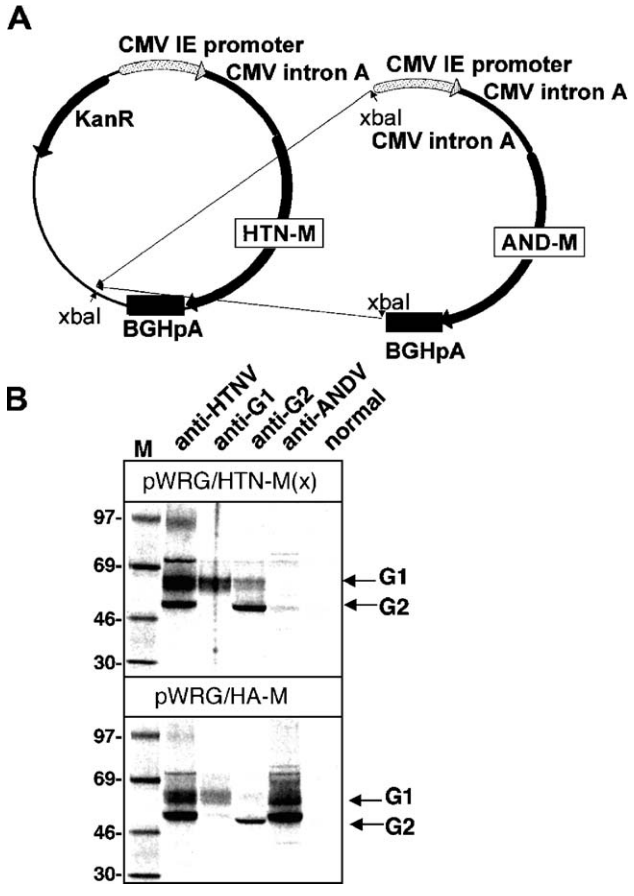

Fig. 1. Construction and expression of pWRG/HA-M DNA vaccine plasmid. The pWRG/HA-M DNA vaccine plasmid was constructed by PCR-amplifying a region of pWRG/AND-M including the CMV promoter, intron A, AND-M genome segment, and bovine growth hormone polyadenylation signal. This fragment was cloned into the pWRG/HTN-M plasmid at the *Xba*I site (A). Expression of both AND-M and HTN-M from pWRG/HA-M was confirmed by RIPA using radiolabeled lysates from COS-7 cells transfected with pWRG/HTN-M (upper panel) or pWRG/HA-M (lower panel) and using HTN-M- and AND-M-specific sera (B). HMAF generated to HTNV detects both G1 and G2, while MAb 6D4 detects HTN-G1 and MAb-HC02 detects HTN-G2. HPS convalescent phase sera detect ANDV G1 and G2. Normal human serum is included as a negative control.

The G1 and G2 glycoproteins of both viruses were expressed from pWRG/HA-M (Fig. 1B).

pWRG/HA-M elicits HFRS and HPS hantavirus neutralizing antibodies in NHPs

To test the immunogenicity of the candidate single-plasmid HFRS/HPS DNA vaccine, two rhesus macaques were vaccinated with pWRG/HA-M four times at 3-week intervals using a gene gun. Sera collected 3 weeks after the fourth vaccination were tested for neutralizing antibodies by PRNT. Serum from NHPs vaccinated with pWRG/HA-M neutralized both ANDV and HTNV (Figs. 2A–B and Table 1). This is the first description of any candidate experimental hantavirus vaccine eliciting neutralizing antibodies against both HFRS and HPS hantaviruses.

To determine the breadth of the neutralizing antibody response, we tested the capacity of the serum to cross-neutralize two North American HPS-associated hantaviruses (SNV and BCCV) and the remaining three HFRS-associated hantaviruses (SEOV, DOBV, and PUUV) (Table 1). We found that each NHP exhibited a different pattern of cross-neutralizing activity. Serum from NHP CAA neutralized three of the seven viruses tested and serum from NHP HJV neutralized five of the seven. Neither NHP produced antibodies that neutralized SEOV or PUUV.

HFRS, HPS, and HFRS/HPS DNA vaccines elicited antibody responses that were readily boosted 1 to 2 years after the initial vaccination series

We monitored the levels of neutralizing antibodies in serum from NHPs vaccinated with pWRG/HA-M and observed that the level of both HTNV and ANDV neutralizing antibodies fell below detection 5 months after the fourth vaccination (Figs. 2A–B). In earlier studies in NHPs, we had reported that the HFRS DNA vaccine (pWRG/HTN-M[x]) and HPS DNA vaccine (pWRG/AND-M) elicited high-titer neutralizing antibodies that were still detectable up to 5 months after the initial vaccination series (Custer et al., 2003; Hooper et al., 2001a) (Figs. 2C–D). To further investigate the duration of immunity elicited by pWRG/HTN-M(x) or pWRG/AND-M, we extended the study and found that homologous neutralizing antibody responses were still detected more than 2 years after the initial vaccination series (Figs. 2C–D). Together, these data indicated that DNA vaccine plasmids containing single hantavirus M genes were capable of eliciting homologous neutralizing antibody titers that were more durable than a single plasmid DNA vaccine containing a combination of two hantavirus M genes.

To determine if the immune responses generated with any of these three DNA vaccines could be readily recalled years after the initial vaccination, we administered a single booster vaccination 1–2 years after the initial vaccination series. For each animal, the booster vaccination consisted of the same DNA vaccine used in the initial series of vaccinations. In all cases, a dramatic increase in neutralizing antibody titers was observed within 11 days after the long-range boost (Figs. 2A–D). In most cases, the neutralizing antibody titers rose to

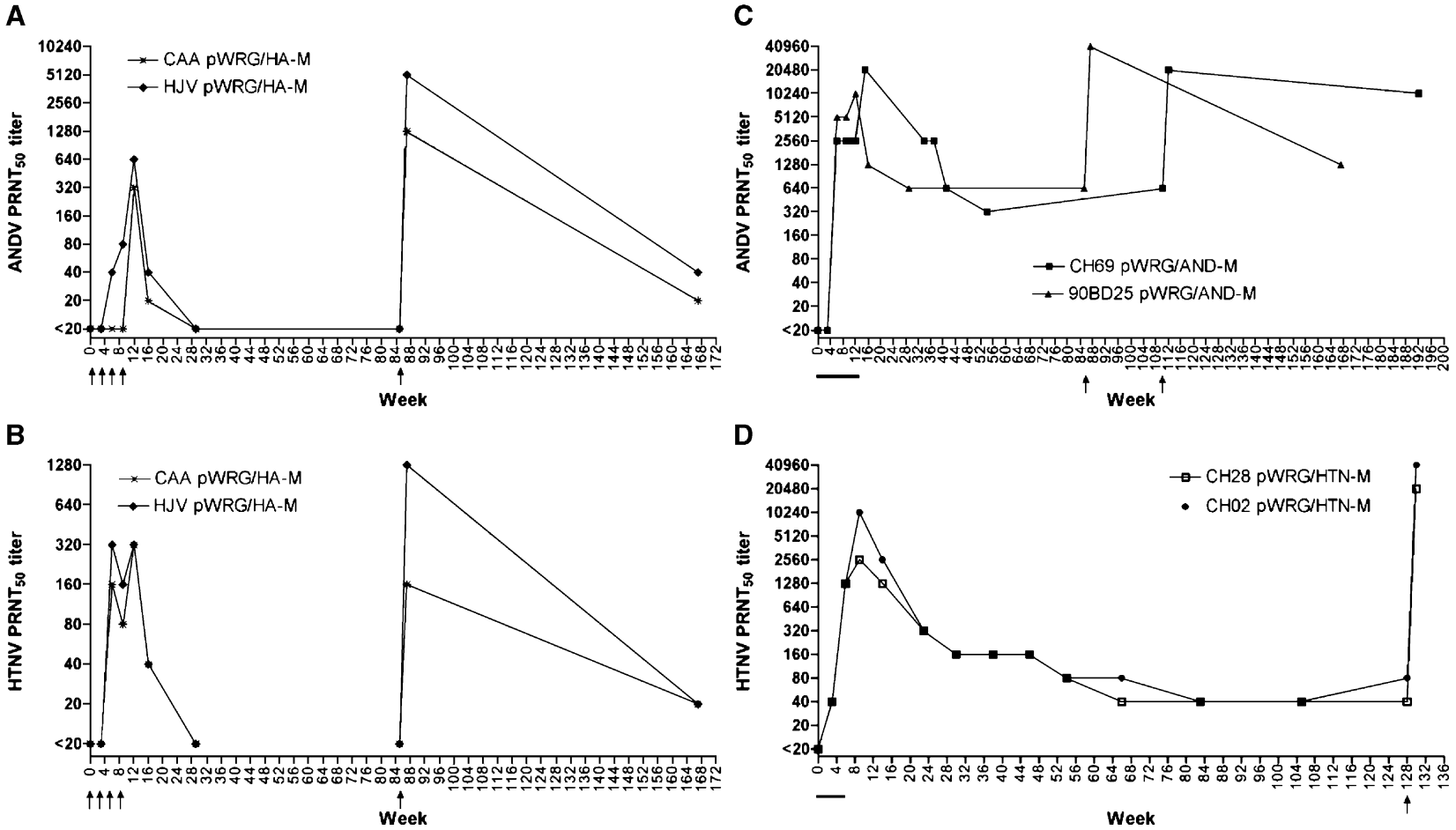

Fig. 2. Immunogenicity of hantavirus DNA vaccines in nonhuman primates. Rhesus macaques were vaccinated with pWRG/HA-M (A and B), pWRG/AND-M (C), or pWRG/HTN-M (D). Neutralizing antibody responses were determined by PRNT after each vaccination and at the indicated weeks. The $PRNT_{50}$ of pWRG/AND-M-vaccinated NHPs to ANDV is shown in panel C. Monkey CH69 was vaccinated on weeks 0, 3, 6, and 12 while monkey 90BD25 was vaccinated on weeks 0, 3, 6, and 9. A long-range booster vaccination was administered at weeks 110 and 85, respectively. NHPs vaccinated with pWRG/HTN-M at weeks 0, 3, 6, and 128 were tested for NAb to HTNV (D). NHP vaccinated with pWRG/HA-M at weeks 0, 3, 6, 9, and 85 was tested for NAb against both ANDV (A) and HTNV (B).

Table 1
pWRG/HA-M elicits neutralizing antibodies in NHP against both HFRS and HPS hantaviruses[a]

Vaccine	Monkey ID	$PRNT_{50}$[b]						
		HPS[c]			HFRS[d]			
		ANDV	SNV	BCCV	HTNV	SEOV	DOBV	PUUV
pWRG/HA-M	CAA	320	<[e]	<	320	<	40	<
	HJV	640	20	20	640	<	20	<
pWRG/AND-M	CH69	20,480*	640*	2560*	<*	<	<	<
	90BD25	10,240*	1280	<	<*	<	<	<
pWRG/HTN-M(x)	CH64	<*	<*	<*	20,480*	160	1280	<
	CH85	<*	<*	<*	20,480*	40	10,240	<
	CH28	<	<	<*	2560*	<*	320*	<*
	CH02	<	<	<*	10,240*	<*	320*	<*

[a] Serum was collected 3 weeks after the final vaccination in the initial series.
[b] Reciprocal of highest dilution neutralizing 50% of the plaques as measured by plaque reduction neutralization test (PRNT) for particular viruses (as indicated).
[c] Viruses associated with hantavirus pulmonary syndrome (HPS).
[d] Viruses associated with hemorrhagic fever with renal syndrome (HFRS).
[e] Less than symbol (<) indicates titer was below detection, <20.
* Data indicated with an asterisk are previously published from other studies and used here as historical data.

similar or even greater levels achieved after the initial vaccination series. These data demonstrated that all three of the hantavirus DNA vaccines elicit potent memory responses.

RIPA performed using pre- and postboost serum from an NHP vaccinated with pWRG/AND-M or pWRG/HA-M indicated that antibodies to both G1 and G2 of ANDV were detected (Fig. 3). The serum from NHPs vaccinated with pWRG/HA-M was more reactive with the ANDV G1 and G2 than the HTNV G1 and G2 (Fig. 3). These biochemical data correlated with the immunogenicity data whereby the ANDV neutralizing antibody titers were 4- to 8-fold greater than HTNV neutralizing antibody titers.

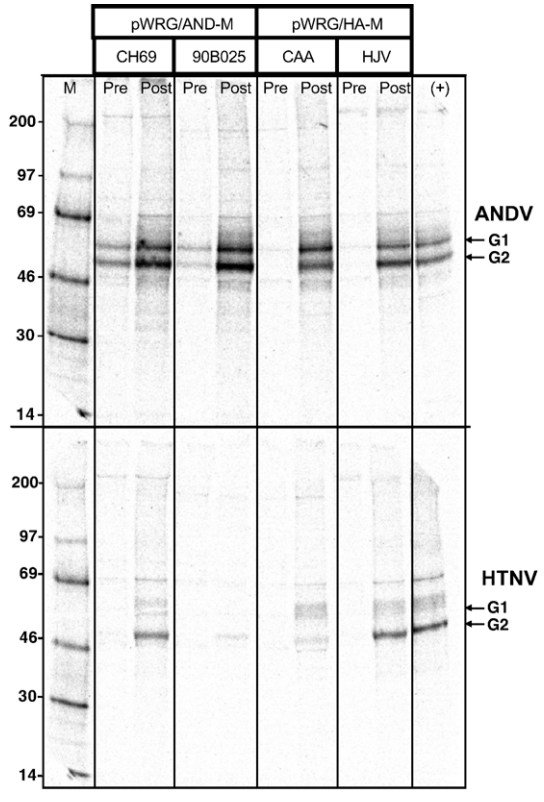

Fig. 3. The immune response to G1 and G2 of DNA vaccines is boostable years after the initial vaccination. The ability to boost G1 and G2 responses in NHP vaccinated with pWRG/AND-M or pWRG/HA-M was determined by collecting preboost (pre) and postboost (post) sera and testing by RIPA for G1- and G2-specific antibodies. Radiolabeled lysates from COS-7 cells transfected with AND-M (A) or HTN-M (B) were reacted with sera of pWRG/AND-M-vaccinated NHP (CH69 and 90BD25) or sera of pWRG/HA-M-vaccinated NHP (CAA and HJV).

Increased capacity to cross-neutralize after long-range booster vaccination

The preboost serum from both NHPs vaccinated with pWRG/AND-M contained detectable levels of G1- and G2-specific antibodies (Fig. 3). This is impressive because one of the NHPs, CH69, was vaccinated 2 years earlier. This same animal was also interesting because its postboost serum immunoprecipitated HTNV G2 (Fig. 3). This was unexpected because serum collected from CH69 3 weeks after the initial vaccination series did not immunoprecipitate HTNV G1 or G2 and did not cross-neutralize HTNV (Custer et al., 2003). This finding suggested that a booster vaccination might result in a qualitatively different antibody response (e.g., greater avidity) with more broadly cross-neutralizing activity. To test this possibility, we performed cross-neutralization PRNT on serum collected after the long-range booster vaccination. The level of cross-neutralizing activity went up in the serum from five of the six NHPs that received long-range booster vaccinations (Table 2). Even NHPs that were vaccinated with the HFRS or HPS DNA vaccine produced antibodies that cross-neutralized HPS- or HFRS-associated hantaviruses, respectively. In fact, the level of cross-neutralizing activity in the NHPs vaccinated with the HFRS or HPS DNA was similar to that observed in the NHPs vaccinated with the dual HFRS/HPS DNA vaccine.

To determine how durable the neutralizing antibody response was after the long-range boost, NHPs were bled 82 weeks after the booster vaccination and PRNT was performed. The memory response elicited by the long-range boost was quite impressive as evidenced by the ability of sera from the

Table 2
Cross-neutralizing activity after long-range booster vaccination

Vaccine	Monkey ID	Duration between prime and boost[a]	$PRNT_{50}$[b]						
			HPS[c]			HFRS[d]			
			ANDV	SNV	BCCV	HTNV	SEOV	DOBV	PUUV
pWRG/HA-M	CAA	76	1280	<[e]	80	160	<	<	<
	HJV	76	5120	160	320	640	320	640	20
pWRG/AND-M	CH69	98	20,480	640	2560	40	40	640	20
	90BD25	76	40,960	1280	320	<	<	40	<
pWRG/HTN-M(x)	CH28	119	20	80	80	20,480	2560	2560	<
	CH02	119	<	<	80	40,960	640	2560	<

[a] Duration between prime and boost is defined as the number of weeks between the final vaccination in the initial series and the long-range boost.
[b] Reciprocal of highest dilution neutralizing 50% of the plaques as measured by plaque reduction neutralization test (PRNT) for particular viruses (as indicated).
[c] Viruses associated with hantavirus pulmonary syndrome (HPS).
[d] Viruses associated with hemorrhagic fever with renal syndrome (HFRS).
[e] Less than symbol (<) indicates titer was below detection, <20.

pWRG/HA-M and pWRG/AND-M-vaccinated NHPs to neutralize virus (Figs. 2A–D). Sera from the pWRG/HA-M-vaccinated NHPs was able to neutralize ANDV, BCCV, and HTNV while sera from the pWRG/AND-M-vaccinated NHPs neutralized only the HPS associated viruses, albeit with exceptionally high titers (Table 3).

pWRG/HA-M does not elicit neutralizing antibodies in hamsters

We previously reported that vaccination with the SEOV or HTNV M segment-based DNA vaccine but not ANDV M segment-based DNA vaccine elicited neutralizing antibodies in Syrian hamsters (Custer et al., 2003). The mechanism underlying the species difference in pWRG/AND-M immunogenicity is not known. To rule out the possibility that a technical error could account for the absence of detectable immunity in hamsters, we performed an experiment where three cynomolgus macaques and eight Syrian hamsters were vaccinated on the same days with pWRG/AND-M gene gun cartridges from the same lot. Animals were vaccinated on weeks 0, 2, 3, and 8. Serum collected from all three cynomolgus macaques contained ANDV neutralizing antibodies: GMT_{50} = 40, 640, and 1280. In contrast, neutralizing antibodies were not detected in any of the eight vaccinated hamsters (data not shown). These data demonstrate that the pWRG/AND-M DNA vaccine administered by gene gun was immunogenic in a second species of macaque (i.e., cynomolgus macaque), and confirms that this plasmid was not immunogenic in Syrian hamsters.

We inoculated hamsters with the pWRG/HA-M vaccine to determine if it was capable of eliciting HTNV and/or ANDV neutralizing antibodies in that species. Eight hamsters were vaccinated with pWRG/HA-M three times at 3-week intervals using a gene gun. Three weeks after the third vaccination, serum was collected and tested for HTNV or ANDV neutralizing antibodies. No HTNV or ANDV neutralizing antibodies were detected in any hamsters (data not shown). In contrast, eight positive control hamsters vaccinated with pWRG/HTN-M(x) developed HTNV neutralizing antibodies ($PRNT_{50}$ GMT = 226; range 20–1280). The hamsters vaccinated with pWRG/HA-M and a group of unvaccinated negative control hamsters were challenged with HTNV. In both groups, seven of eight hamsters were not protected from infection because they demonstrated serological evidence of infection. Thus, the ANDV M genome segment contained in pWRG/HA-M was not only nonimmunogenic in hamsters (when delivered by gene gun), but it also exerted a dominant-negative effect on the immunogenicity of the HTNV M genome segment component of the vaccine.

Table 3
Neutralizing antibody memory response elicited after a long-range booster vaccination

Vaccine	Monkey ID	Weeks after boost until bleed[a]	$PRNT_{50}$[b]						
			HPS[c]			HFRS[d]			
			ANDV	SNV	BCCV	HTNV	SEOV	DOBV	PUUV
pWRG/HA-M	CAA	82	20	<[e]	<	20	<	<	<
	HJV	82	40	<	20	20	<	<	<
pWRG/AND-M	CH69	82	10,240	160	320	<	<	<	<
	90BD25	82	1280	160	20	<	<	<	<

[a] Duration between boost and bleed is defined as the number of weeks between the long-range booster vaccination and the final bleed.
[b] Reciprocal of highest dilution neutralizing 50% of the plaques as measured by plaque reduction neutralization test (PRNT) for particular viruses (as indicated).
[c] Viruses associated with hantavirus pulmonary syndrome (HPS).
[d] Viruses associated with hemorrhagic fever with renal syndrome (HFRS).
[e] Less than symbol (<) indicates titer was below detection, <20.

Discussion

We previously reported that candidate HFRS and HPS DNA vaccines administered by gene gun elicit high-titer neutralizing antibodies in NHPs (Custer et al., 2003; Hooper et al., 2001a). Thus, a DNA vaccine comprised of these two plasmids (expressing the HTNV and ANDV M genes, respectively) could be considered a candidate HFRS/HPS vaccine. Here, we have explored an alternative strategy whereby dual hantavirus M genes were combined into a single plasmid. One advantage of this approach is that it simplifies downstream production processes and reduces costs because there are fewer drug substances (plasmids) to manufacture and characterize. The use of a bi- or multi-immunogen plasmid can alleviate some of the drawbacks associated with a multiplasmid approach provided that there are sufficient immune responses elicited against each of the target immunogens. Previous reports suggest that it is possible to actually improve immunogenicity by using dual-immunogen constructs as compared to administration of separate plasmids. For example Song et al. (2005) reported the generation of a DNA vaccine plasmid expressing measles virus hemagglutinin (MV-H) and fusion (MV-F) proteins. A decrease in MV-specific serum PRNT titers and avidity in mice vaccinated with dual-immunogen constructs was observed; however, the mice showed increases in IFN-gamma producing MV-specific splenocytes compared to mice vaccinated with MV-H or MV-F on separate plasmids. Several groups have used a bicistronic DNA vaccine approach to combine an immunogen-of-interest with cytokines or co-stimulatory molecules (e.g., interleukin-18 or GM-CSF) on the same plasmid (Chang et al., 2004; Lee et al., 1998). In those studies, immunogenicity to the vaccine immunogen-of-interest was improved relative to co-administration of separate plasmids. To date, the use of dual-immunogen plasmids has only been used to improve the immunogenicity of vaccines against single agents (Song et al., 2005). pWRG/HA-M is the first multiagent DNA vaccine plasmid, to our knowledge, that expresses target genes from different virus species (i.e., HTNV and ANDV).

Immunogenicity of pWRG/HA-M

NHPs vaccinated with pWRG/HA-M developed antibody responses that neutralized both HTNV and ANDV; however, the endpoint titers were several-fold lower than those achieved with plasmids expressing only the HTNV or ANDV glycoproteins (Fig. 2 and Table 1). Nevertheless, the titers elicited by pWRG/HA-M were respectable, especially in light of the fact that the titers of neutralizing antibody generated by the hantavirus M gene-based DNA vaccines pWRG/HTN-M(x) and pWRG/AND-M (as high as $PRNT_{50}$ = 40,960) were among the highest levels of neutralizing antibodies reported for any DNA vaccine to any viral antigen delivered to NHPs by gene gun. These high-titer antibody responses, which are similar to those produced in persons who survive HPS using the same assay (unpublished data), demonstrate that potent neutralizing antibody responses can be achieved using a DNA vaccine approach that requires neither adjuvant nor protein boosts.

Duration of immunity

The neutralizing antibody response elicited by the dual-immunogen DNA vaccine in this study was less durable than that elicited by the single-immunogen vaccines. Two months after the initial vaccination series with pWRG/HA-M, neutralizing antibodies against both HTNV and ANDV were detected; however, at the next time point (3 months later), neutralizing antibody levels were below detection. In contrast, the single-immunogen vaccines elicited neutralizing antibody responses that were still detectable 1–2 years after the initial vaccination series (Figs. 2C–D). In all of the NHPs in Fig. 2, there was an approximately 8- to 64-fold reduction in neutralizing antibody titers during the first 6 months after the first vaccination. A >16-fold reduction in antibodies would result in a PRNT titer below detection for the pWRG/HA-M-vaccinated NHPs because their peak titers were between 320 and 640 after the initial vaccination series. Thus, the reduced duration of detectable levels of antibody in the serum of NHPs vaccinated with pWRG/HA-M was likely related to the reduced potency of the initial response.

Long-range boost

Perhaps one of the most significant aspects of this study is the finding that a single DNA vaccine booster vaccination 1–2 years after the initial series results in a rapid and potent rise in levels of neutralizing antibodies. In most cases, the titers achieved after this long-range boost were greater than the peak titers after the initial vaccination series. This rapid recall of neutralizing antibodies was detected in all eight NHPs that received a booster vaccination. Unlike the initial pWRG/HA-M series, the capacity of the sera to neutralize after the long-range boost did not drop below detection after more than 1.5 years. Longevity of memory response has also been reported after DNA vaccination (via gene gun) of NHPs with a plasmid encoding the rabies virus glycoprotein. In this study, neutralizing antibodies could be detected up to 588 days after the initial vaccination (Lodmell et al., 2002).

The rapid recall response observed after long-range booster vaccination could play a role in the efficacy of the vaccine. We speculate that if a vaccinated person with a waning neutralizing antibody response was exposed to a hantavirus, then the resulting infection would trigger the same rapid rise in neutralizing antibodies observed in NHPs that received a long-range booster vaccination. If this were true, then these neutralizing antibodies would rise to protective levels before the virus could proceed through the 1–2 week hantavirus incubation period. Bharadwaj et al. reported a correlation between the level of neutralizing antibodies at the time of hospital admission and severity of disease. Patients with higher levels of neutralizing antibodies developed mild disease whereas patients with lower levels of neutralizing antibodies

developed severe HPS (Bharadwaj et al., 2000). We demonstrated that hamsters challenged with a lethal dose of ANDV were protected against disease if injected with ANDV neutralizing antibodies (produced in NHPs vaccinated with pWRG/AND-M) up to 5 days after challenge (Custer et al., 2003). These findings support the concept that maintenance of high-titer neutralizing antibodies might not be necessary to confer protection as long as a rapid recall of a neutralizing antibody response occurs upon hantavirus exposure.

Cross-reactivity

One of our main goals is to develop a vaccine that protects against multiple hantaviruses. Hence, we are interested in evaluating the capacity of candidate vaccines to cross-neutralizing (and presumably cross-protect) heterologous hantaviruses. Convalescent sera from HFRS and HPS patients have demonstrated a variable capacity to cross-neutralize heterologous hantaviruses (Chu et al., 1995). In addition, numerous reports on the evaluation of experimental HFRS vaccines have described varying degrees of cross-neutralizing antibody responses against HFRS-associated viruses (Hooper and Li, 2001). In our previous studies, pWRG/HTN-M(x) elicited antibodies that could neutralize three of the four HFRS hantaviruses (i.e., HTNV, SEOV, DOBV), but not PUUV or any of the HPS hantaviruses tested (Custer et al., 2003; Hooper et al., 2001a). Conversely, pWRG/AND-M elicited antibodies that could neutralize other HPS hantaviruses, but not HFRS hantaviruses (Custer et al., 2003). Here, the NHPs vaccinated with pWRG/HA-M developed neutralizing antibody responses against both HPS and HFRS hantaviruses after the 4th vaccination. This is the first time any vaccine has elicited neutralizing antibodies against both HFRS and HPS hantaviruses. Interestingly, the capacity of not only pWRG/HA-M but also pWRG/HTN-M(x) and pWRG/AND-M to cross-neutralize heterologous hantaviruses increased after the long-range booster vaccination. In fact, after the long-range boost, two NHPs (HJV vaccinated with pWRG/HA-M and CH69 vaccinated with pWRG/AND-M) developed antibodies that neutralized all seven of the HPS and HFRS hantaviruses in our panel. This finding indicates that a long-range booster vaccination not only extends the duration of protective immunity, but also might increase the breadth of cross-protective immunity to include known and unknown hantaviruses that cause disease in humans. We speculate that the increased breadth in cross-reactivity is due to an increase in the avidity of antibodies that bind neutralizing epitopes that are similar but not identical among the different species of hantaviruses. As yet, we have not attempted to identify neutralizing epitopes or measure the avidity of the antibodies contained in the polyclonal immune serum generated by these DNA vaccines. Others have reported that acute-phase serum from HPS patients has an increased capacity to cross-neutralize (Ye et al., 2004). The observation that a long-range booster vaccination increases the breath of cross-neutralizing activity suggests that future vaccination protocols involving hantavirus M gene-based technologies should consider long-range boost components to raise the levels of neutralizing and cross-neutralizing antibodies and to extend the duration of immunity.

Species specificity

The HTNV and SEOV M gene-based DNA vaccines were immunogenic in both hamsters and NHPs; however, the ANDV M gene-based DNA vaccine and pWRG/HA-M were immunogenic in NHPs but, inexplicably, not in hamsters. The ANDV glycoproteins apparently have a dominant negative effect on the immunogenicity of the HTNV glycoproteins when they are combined on the same plamid because hamsters vaccinated with this plasmid did not develop neutralizing antibodies to HTNV. This effect could be due to interactions between the recombinant HTNV and ANDV glycoproteins (e.g., hetero-oligomers) that render the complexes invisible to the hamster immune system. For example, hetero-oligomers might be improperly targeted and/or rapidly degraded before being presented to the hamster immune system. The capacity of glycoproteins from heterologous hantaviruses to interact when co-expressed in the same cells has been reported previously (Deyde et al., 2005). Alternatively, the ANDV glycoproteins might somehow down-regulate the hamster's immune response to the expressed glycoproteins, and collaterally down-regulate the response to the co-expressed HTNV glycoproteins. Hamsters infected with ANDV develop HPS and succumb after approximately 10–12 days postchallenge but survivors do produce ANDV-specific neutralizing antibodies indicating that hamsters can develop anti-ANDV neutralizing antibodies under the appropriate conditions (Hooper et al., 2001b). It is tempting to speculate that the inability of pWRG/AND-M or pWRG/HA-M to elicit neutralizing antibodies is related to the exceedingly high virulence of ANDV in hamsters. For example, the ANDV glycoproteins could function to down-regulate the immune response in hamsters, and this delay could then allow dissemination of the virus with fatal consequences. At this point in time, however, we have no evidence supporting such a relationship, and the two phenomena could be completely unrelated.

In summary, we demonstrated that it is possible to elicit neutralizing antibody responses against both HFRS and HPS hantaviruses using a single plasmid, dual-immunogen, DNA vaccine administered by gene gun. In addition, we demonstrated that hantavirus M gene-based DNA vaccines elicited potent immunological memory and extraordinarily high levels of neutralizing antibodies were rapidly produced after a single long-range booster vaccination. The production-associated benefits of combining multiple immunogens into a single plasmid might be out-weighed by a loss in overall potency. Here, we elicit neutralizing antibody titers that were 8- to 32-fold lower in the dual-immunogen plasmids than in the single-immunogen plasmids, albeit the number of vaccinated animals was very low. Future investigations will include experiments to determine if vaccination with a combination of single-immunogen hantavirus M gene-based plasmids can elicit

neutralizing and cross-neutralizing antibody responses greater than those achieved with pWRG/HA-M.

Materials and methods

Viruses, cells, and antibodies

HTNV, strain 76–118 (Lee et al., 1978), SEOV, strain SR-ll (Kitamura et al., 1983), DOBV (Avsic-Zupanc et al., 1992), PUUV, strain K27 (Tkachenko et al., 1984), ANDV strain Chile-9717869 (Hooper et al., 2001b), BCCV (Rollin et al., 1995), and SNV strain CC107 (Schmaljohn et al., 1995) were propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586). Transient expression experiments were performed with COS-7 cells (COS-7; ATTC CRL1651). Both cell types were maintained in Eagle's minimal essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum (FBS), 10 mM HEPES pH 7.4, and antibiotics (penicillin [100 U/ml], streptomycin [100 μg/ml], and gentamicin sulfate [50 μg/ml]) (cEMEM) at 37 °C in a 5% CO_2 incubator.

Immunoprecipitation

Radio-immunoprecipitation experiments were performed using the HTNV G2-specific monoclonal antibody (MAb)-HC02 (provided by Dr. J. McCormick of the Centers for Disease Control, Atlanta, GA), the HTNV G1-specific MAb-6D4, hyperimmune mouse ascetic fluid (HMAF) generated to HTNV (Wang et al., 1993), or HPS convalescent phase sera from Argentina, as described previously (Custer et al., 2003).

Construction of Hantaan/Andes dual M genome segment-based DNA vaccine plasmid

Construction of the HTNV M DNA vaccine plasmid, pWRG/HTN-M(x), and ANDV M DNA vaccine plasmid, pWRG/AND-M, was described previously (Custer et al., 2003; Hooper et al., 2001a). To construct a plasmid expressing both the HTNV and ANDV glycoproteins, a fragment from pWRG/AND-M containing the CMV promoter, intron A, ANDV M genome segment, and bovine growth hormone polyadenylation signal was PCR-amplified by using the following primers: forward primer [5′-GGC CTC TAG AGC AGG TCG ACA ATA TTG GCT ATT GGC C] and reverse primer [5′-GCG CAA TTC TAG AGT CGA GCT GTC GAG CCC C]. Both primers included an *Xba*I restriction site. The PCR reactions were performed with Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA): one 3-min cycle at 94 °C followed by 30 cycles of 94 °C 30 s, 68 °C 8 min. The PCR product was cut with *Xba*I and then ligated into the *Xba*I site of pWRG/HTN-M(x) using T4 DNA ligase (New England Biolabs, Beverly, MA). The resulting dual-immunogen plasmid was designated pWRG/Hantaan/Andes-M (19a), or pWRG/HA-M. Plasmid DNA was purified using Qiagen maxiprep DNA purification kits according to the manufacturer's directions. To confirm the identity of the new construct, the HTNV and ANDV M genes contained in pWRG/HA-M were sequenced using an ABI 3100 genetic analyzer.

Immunoprecipitation

Radio-immunoprecipitation assays (RIPA) using COS cell lysate labeled with Promix ([^{35}S]-methionine and [^{35}S]-cysteine, Amersham, Piscataway, NJ) were performed essentially as described previously (Hooper et al., 2001a). Protein G-Sepharose (Sigma, St. Louis, MO) was used in place of protein A. Reduced samples were run on 4 to 12% gradient Tris–Bis Nupage Gels using 1× MOPS as a running buffer with 1× MOPS-0.25% (vol/vol) antioxidant in the inner chamber, at 200 V constant voltage. Digital images were obtained using a Cyclone phosphoimager (Packard, Meridan, CT).

Gene gun vaccinations

Cartridges for the gene gun were prepared as described previously (Hooper et al., 1999, 2001a). Gene gun cartridges consisting of 1 μg of plasmid DNA coated on 0.5 mg of gold were prepared and stored at 4 °C, desiccated, until use. Female rhesus macaques (*Macaca mulatta*) were vaccinated with the Powderject XR1 particle-mediated epidermal delivery device (gene gun) (PowderMed, LTD), with four administrations at nonoverlapping sites on the shaved abdomen and four over inguinal lymph nodes using 400 lbs/inch^2 of helium pressure. Female Syrian hamsters (*Mesocricetus auratus*) more than 8 weeks old were vaccinated with the same type of cartridges and the same gene gun conditions used to vaccinate the NHP; however, the hamsters received four administrations (on abdomen) per vaccination, rather than eight. Animals were anesthetized during the nonpainful gene gun procedure. The only visible effect was mild erythema at the vaccination sites.

Plaque-reduction neutralization tests (PRNT)

Neutralization assays were performed as previously described (Hooper et al., 2001a). Serum samples were heat inactivated and 5% guinea pig complement (Accurate Chemical and Scientific Corp., Westbury, NY) was included in the assay. HTNV, ANDV, SEOV, and BCCV PRNT were stained with neutral red after 7 days; and PUUV, DOBV, and SNV PRNT were stained after 10 days. Plates were incubated at 37 °C and plaques counted 2–3 days after staining.

N-specific ELISA

The enzyme-linked immunosorbent assay (ELISA) used to detect antibodies that bind recombinant truncated hantavirus N protein was described previously (Custer et al., 2003; Elgh et al., 1997; Hooper et al., 1999).

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